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T cell senescence predicts subclinical atherosclerosis in HIV-infected patients similarly to traditional cardiovascular risk factors

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ABSTRACT

The main objective of this study is to evaluate the predictive capacity of T cell activation/senescence in subclinical atherosclerosis (SCA) in a group of HIV-infected patients. So, a cross-sectional analysis was performed on 91 long-term triple-ART therapy HIV-infected patients from an observational and prospective cohort. Carotid Intima Media Thickness (cIMT) was measured. Binary logistic regression was used to evaluate independent variables associated with SCA. Compared to patients without SCA, patients with SCA (60.4%) were older (41.33 ± 9.04 vs. 51.73 ± 8.44 years old, $p < 0.001$) and showed Framingham risk score (2.63 ± 3.127 vs. 7.66 ± 5.84, $p = 0.008$), as well as higher numbers of $CD4^+CD8^+$ double positive T cells (0.50 ± 0.42% vs. 0.81 ± 0.79%, $p = 0.037$), $CD8^+CD28^-$ T cells (41.70 ± 16.96% vs. 50.22 ± 16.15%, $p = 0.018$), higher expression of CD28 on $CD8^+CD28^+$ T cells (1865 ± 789 vs. 2243 ± 917 MFI, $P = 0.046$). In contrast, they showed lower expression of CD38 on $CD19^+$ B cells (65.38 ± 27.47% vs. 42.67 ± 30.26%, $P < 0.001$). Logistic multivariable analysis showed that Framingham risk score > 10% (OR = 14.84, CI95% 1.63–125; $p = 0.016$) and numbers of $CD8^+CD28^-$ T cells (OR = 1.032, CI 95% 1–1.065; $p = 0.045$) were independent factors associated with SCA. Patients with $CD8^+CD28^-$ T cells ≥59% compared to those < 59% had higher risk of SCA (OR = 4, CI95% 1.19–13.3, $p = 0.024$). Interestingly, 27.4% of patients with low Framingham risk score had elevated levels of $CD8^+CD28^-$ T cells. In conclusion, immune senescence represented by accumulation of $CD8^+CD28^-$ T cells may contribute to improve the predictive capacity of the Framingham risk score, especially when the scores are low and can explain, at least in part, the higher prevalence of SCA observed in long-term ART-treated stable HIV infected patients.

1. Introduction

Life expectancy of HIV-infected patients has considerably increased due to the introduction of antiretroviral treatments (ART). However, morbidity and mortality are still higher in these patients when compared with general population (Marcus et al., 2016; Rasmussen et al., 2015). At present, the most frequent causes of death in HIV patients are the so-called non-AIDS events, which include cardiovascular and neurodegenerative diseases, kidney failure, liver diseases, osteoporosis and non–AIDS-defining cancers (Masiá et al., 2013). Among them, atherosclerosis associated cardiovascular disease (CVD), including myocardial infarction and stroke, is currently one of the main causes of mortality among HIV- patients (Bloomfield and Leung, 2017). Despite being today a focus of intensive research, CVDs pathophysiology associated to HIV infection is mostly unknown. Although classic risk factors can interact, some of them more frequently than in general population, such as tobacco or dyslipidemia (Masiá et al., 2012), we cannot rule out a direct damage of the virus at the endothelial level, a toxic effect of ARTs, or even an impact of the mechanisms related to immune alteration associated with HIV latency (Kearns et al., 2017). Ultimately, the common pathogenic link is endothelial dysfunction as a precursor to development of atherosclerosis and appearance of cardiovascular events (Masiá et al., 2010).

Although CVDs pathogenesis is extraordinarily complex, the immune system seems to play key roles. Chronic inflammation is well accepted as a cardiovascular risk factor in general population (Recio-Mayoral et al., 2009; Libby, 2008). Ageing is associated with a state of chronic low-grade inflammation known as “inflammaging”, which seems to be

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involved in most age-related diseases (Franceschi and Campisi, 2014). Besides, immune senescence is characterized by decreased output of naïve T cells following thymic involution, and accumulation of highly differentiated CD28 null ($CD28^-$) memory T cells, as a result of repeated antigenic stimulation and chronic viral infections such as cytomegalovirus (CMV) (Arnold et al., 2011). In HIV-infected patients, immune senescence can be accelerated due to the immune activation maintained over time that can appear together with viral reactivation (HIV, CMV) and/or gastrointestinal bacterial translocation (Fletcher et al., 2005; Appay et al., 2007). Recently, it has been shown that immune senescence appears to be highly dependent on HIV-infection and only to a smaller extent associated with age-related parameters in well-treated HIV-infected patients (Tavenier et al., 2015). In virally suppressed HIV-infected patients, or even in HIV elite controllers without measurable viremia, abnormally high $CD38^+HLA\text{-}DR^+$ activated T cell numbers are detected, which might contribute to the initiation of endothelial activation and subsequent atherogenesis (D'Abramo et al., 2014). Additionally, B-cell abnormalities that are associated with HIV-replication-induced immune cell activation, particularly loss of memory B-cells and the decrease in memory B-cell function, which persist even after several years of effective ART, could further contribute to the immune activation state in HIV suppressed patients due to a less efficient control of concomitant infections (Moir and Fauci, 2008).

Nowadays, the role of immune activation and immune senescence in the pathogenesis of atherosclerosis is controversial. While some studies appear to relate these immunological parameters to a higher probability of subclinical atherosclerosis (D'Abramo et al., 2014; Kaplan et al., 2011), in others, this association has not been observed (Grome et al., 2017; Guaraldi et al., 2013; Merlini et al., 2012).

For this reason, the main objective of this study is to evaluate the predictive capacity of immune activation/immune senescence in subclinical atherosclerosis in a group of HIV-infected patients receiving long-term ART with low viral load, and to analyze the associated factors. The influence of classical cardiovascular and thrombosis risk factors will be evaluated.

2. Material and methods

2.1. Study design, participants, setting and eligibility

A cross-sectional analysis was performed on a prospective and observational cohort of HIV-infected patients. We analyzed patients who attended university-based HIV clinic in Murcia, Spain, between February 2015 and June 2016. Subjects were recruited if they were HIV-infected on stable triple ART, defined as continuous treatment with three antiretroviral drugs including either NNRTI-based regimens (2 nucleoside reverse transcriptase inhibitor [NRTI] and 1 non-nucleoside reverse transcriptase inhibitor {NNRTI]), PI-based regiments (2 NRTI and 1 protease inhibitor-boosted with ritonavir [PI/r]), IIS-based regimens (2 NRTI and 1 integrase strand transfer inhibitor [INSTIs]), and if they had had plasma HIV RNA lower than 200 copies/ml for at least six months.

Exclusion criteria included the presence of CVDs (previous stroke, myocardial infarction or intermittent claudication). The study conformed to the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines and was approved by the local Ethics Committee ("Comité Ético de Investigación Clínica del Hospital General Universitario Reina Sofía de Murcia"). All patients gave their written informed consent to participate in the study.

2.2. Clinical and laboratory measurements

Medical records were carefully reviewed and all subjects underwent a physical examination. Information on gender, age, body mass index, smoking status, family history of CVDs, and treatment with antiretroviral drugs was recorded. The presence of arterial hypertension, hypercholesterolaemia and hypertriglyceridaemia was defined according to the Adult Treatment Panel III criteria (National Cholesterol Educ, 2002). A sample of fasting venous blood was obtained to determine concentrations of glucose, high-sensitivity C-reactive protein (hsCRP), creatinine, total cholesterol, D-Dimer, high-density lipoprotein (HDL) cholesterol and triglycerides using standard enzymatic methods. Low-density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald equation. Plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Branchburg,NJ). CD4 and CD8 T cell counts were determined by flow cytometry (Beckman-Coulter, Münster, Germany). Plasma levels of hsCRP were measured using nephelometry (Siemens Healthcare Diagnostics, Deerfield, IL).

2.3. Flow cytometric analysis of activation and senescence biomarkers

EDTA anticoagulated peripheral blood cells were labeled following a lyse/wash protocol with an 8-color/9-monoclonal antibody (mAb) panel including CD3 AmCyam (clone SK7, BD Biosciences), CD4 PECy7 (SK3, BD), CD8 APCCy7 (SK1, BD), CD16 PacBlue (3G8, BD), CD19 PECy7 (SJ25C1, BD), CD28 FICT (CD28.2, BD), CD38 APC (HB7, BD), CD86 PE (IT2.2, BD) and HLA-DR PerCP (L243, BD). Five microliters of each antibody in 100 µl of whole blood were incubated for 15 min at room temperature in the dark. Samples were lysed with 3 ml of 1X FACSlysing solution (BD) for 5 min and washed with 3 ml of FACSFlow (BD). Half a million cells were immediately acquired in a FACSCanto flow cytometer (BD), daily calibrated using 7-color setup beads (BD), and analyzed with DIVA software (BD) following the gating strategy described in Fig. 1.

The expression of $CD28^+$, $CD38^+$, $CD86^+$, and $HLA\text{-}DR^+$ activation/senescence markers were evaluated as percentage or absolute numbers (cells/µl) of positive cells as well as mean fluorescence intensity (MFI) of the marker on $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD4^+CD8^+$ T lymphocytes, $CD19^+$ B lymphocytes and $CD3^-CD19^-CD16^+$ NK lymphocytes, Monocytes ($CD4^+CD86^+HLA\text{-}DR^+$ medium SSC cells), granulocytes ($CD16^{++}$ elevated SSC cells) and eosinophils (elevated SSC auto fluorescent cells).

2.4. cIMT measurements

Carotid measurements were performed at the baseline visit. To determine the cIMT, B-mode high resolution ultrasound was used following a standard procedure previously described (Bernal Morell et al., 2016). All measurements were performed by the same investigator, who was blinded to the group to which the patients belonged. It was considered subclinical atherosclerosis if IMT was higher than 0.8 mm in common carotid, higher than 1 mm in bulb carotid or there was a plaque in the carotid artery.

2.5. Statistical analyses

A descriptive analysis of patients' characteristics was carried out using frequency tables for categorical variables. Mean and standard deviation (SD) were used for continuous variables. Differences in categorical variables between patients with and without subclinical atherosclerosis were assessed through the chi-squared test or Fisher test, and t-student tests for continuous variables. Binary logistic regression was used to evaluate the independent variables associated with Subclinical atherosclerosis. Multivariable models were adjusted for sex, age, transmission group (homo/bisexual, injecting drug use, heterosexual, other/unknown), ART regimen (NNRTI-, PI-, IIS-based regimens, other/non-specified), HIV viral load, and Framingham risk score higher or lower than 10%. Wald tests were used to derive p-values.

Significance levels were placed at $p < 0.05$. All statistical analyses were performed using SPSS package version 22.

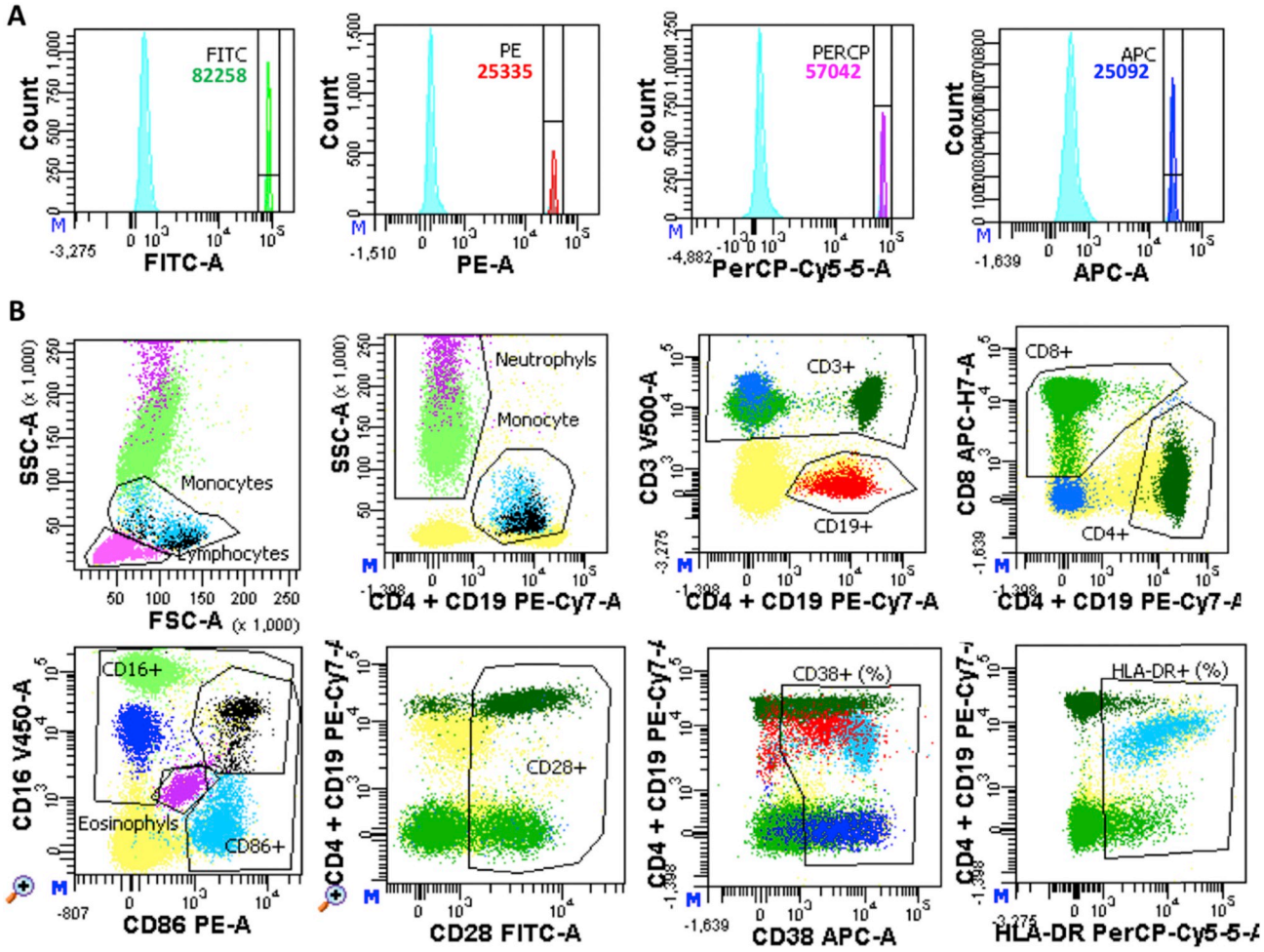

Fig. 1. Analysis of activation and senescence markers in peripheral blood cells. A) BD FACSCanto-II flow cytometer was daily calibrated using 7-color setup beads (BD) to set voltage for FITC, PE, PerCP and APC detectors to the target values fixed at the beginning of the study. These detectors correspond to CD28, CD86, HLA-DR and CD38 markers, respectively. Fluorescent compensations were adjusted manually to set mean fluorescence intensity (MFI) of positive populations at similar values to those of the internal negative control cells. B) Gating strategy to analyze the expression (percentages and MFI) of CD28, CD38, CD86 and HLA-DR activation markers on $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD3^+CD4^+CD8^+$ T lymphocytes, $CD19^+$ B lymphocytes and $CD3^-CD19^-CD16^+$ NK lymphocytes, as well as on Monocytes ($CD4^+CD86^+HLA\text{-}DR^+$ medium SSC cells), granulocytes ($CD16^{++}$ elevated SSC cells) and eosinophils (elevated SSC auto fluorescent cells).

3. Results

A total of 91 HIV-infected patients, with 109 ± 104 months of HIV infection duration, were included. The median age was 47 ± 10 years, 77% male, median $CD4^+$ T cell was 748 ± 318 cells/ml and 85% had HIV-viral load lower than 50 copies/ml. Treatment consists on PI-based regiments (51.1%), NNRTI-based regimens (33.3%) and IIS-based regimens (16%). Hypertension (18.7%), type-2 diabetes (7.7%) and dyslipidemia (20%) were observed, 43.8% of patients were smokers. The mean Framingham risk score was 5.7 ± 5.5, with 18% of patients showing Framingham risk scores higher than 10%.

Table 1 summarizes demographics and the main clinical characteristics of patients distributed by subclinical atherosclerosis, 60.4% of patients included had subclinical atherosclerosis. Compared to patients without atherosclerosis, patients with subclinical atherosclerosis were older (41.33 ± 9.04 vs. 51.7 ± 8.44 years old, $p < 0.001$), more likely to be hypertensive (5.6% vs. 27.3%, $p = 0.02$), and showed higher levels of total cholesterol (181.29 ± 44.56 vs. 202.57 ± 45.54 mg/dl, $p = 0.037$), Framingham risk score (2.63 ± 3.127 vs. 7.66 ± 5.84, $p = 0.008$), and higher probability of Framingham risk score more than 10% (2.9% vs. 27.8%, $p = 0.003$). Besides, they showed higher viral load (27 ± 20 vs. 37 ± 38 copies/ml, $p = 0.043$), cIMT left bulb carotid (0.72 ± 0.13 vs. 1.09 ± 0.33 mm, $p < 0.001$), and cIMT left common carotid (0.58 ± 0.10 vs. 0.77 ± 0.19 mm, $p < 0.001$).

Table 2 summarizes the immunological parameters distributed by subclinical atherosclerosis. Compared to patients without atherosclerosis, patients with subclinical atherosclerosis showed higher numbers of $CD4^+CD8^+$ double positive T cells (0.50 ± 0.42% vs. 0.81 ± 0.79%, $P = 0.037$), $CD8^+CD28^-$ T cells (41.70 ± 16.96% vs. 50.22 ± 16.15%, $P = 0.018$), higher expression of CD28 receptor on $CD8^+CD28^+$ T cells (1865 ± 789 vs. 2243 ± 917 MFI (mean fluorescence intensity), $P = 0.046$), and higher expression of CD8 receptor on NK cells (24.87 ± 14.71% vs. 32.30 ± 17.99%, $P = 0.042$). Instead, patients with subclinical atherosclerosis showed lower numbers of $CD8^+CD28^+$ T cells (58.29 ± 16.95% vs. 49.78 ± 16.17%, $P = 0.018$) and lower expression of CD38 receptor on $CD19^+$ B cells (65.38 ± 27.47% vs. 42.67 ± 30.26%, $P = 0.001$).

Logistic regression models were used to analyze the association between subclinical atherosclerosis and clinical and immunologic variables. The multivariable analysis showed that Framingham risk score higher than 10% (OR = 14.84, CI95% 1.63–125; $p = 0.016$) and numbers of $CD8^+CD28^-$ T cells (OR = 1.032, CI 95% 1–1.07; $p = 0.045$) were independent factors associated with subclinical atherosclerosis (Fig. 2 and Table 3).

The frequency of $CD8^+CD28^-$ T cells was categorized according to the 75th percentile of the population, i.e. patients with $CD8^+CD28^-$ T cells ≥59% vs. < 59%. From this analysis, it was observed that patients with higher frequency of $CD8^+CD28^-$ T cells had higher risk of

Table 1
Patient characteristics distributed by subclinical atherosclerosis.

Parameters (mean ± sd or %)	No atherosclerosis N = 36 (39.5%)	Subclinical atherosclerosis N = 55 (60.4%)	p
Demographic and clinical			
Age years	41.33 ± 9.04	51.73 ± 8.44	< **0.001**
Sex Male, n (%)	27 (75.0)	43 (78.2)	0.922
Body mass index, kg/m2	26.17 ± 5.99	26.37 ± 4.35	0.901
Lipodystrophy, n (%)	3 (9.7)	5 (11.9)	1.000
Hepatitis C virus ab, n (%)	5 (14.3)	15 (27.8)	0.219
Hepatitis B virus antibodies, n (%)	3 (12.5)	4 (19.0)	0.847
Blood and urinary analysis			
Glucose mg/dl	104.51 ± 53.17	99.46 ± 20.19	0.529
Total cholesterol mg/l	181.29 ± 44.56	202.57 ± 47.54	**0.037**
LDL-cholesterol mg/dl	107.17 ± 41.76	122.89 ± 38.38	0.072
Triglycerides mg/dl	148.29 ± 118.08	161.31 ± 84.18	0.545
HDL-cholesterol, mg/dl	47.00 ± 15.25	50.41 ± 13.57	0.273
C-Reactive protein mg/dl	0.43 ± 0.59	0.33 ± 0.48	0.544
Glomerular filtration Rate (mL/min)	96.19 ± 7.46	95.83 ± 14.10	0.914
Albuminuria (mg/g)	10.76 ± 17.92	15.95 ± 27.84	0.459
D-dimer (mg/dl)	280.26 ± 198.53	400.27 ± 541.03	0.214
Parameters related to HIV infection			
Time on ART, months	94.47 ± 83.43	119.08 ± 115.71	0.290
AIDS diagnosis, n (%)	7 (19.4)	7 (12.5)	0.531
T lymphocyte nadir cells/ml	264.22 ± 199.16	229.85 ± 142.34	0.340
HIV viral load copies/ml	27 ± 20	37 ± 38	**0.043**
NNRTI, n (%)	16 (45.7)	15 (28.3)	0.148
Protease Inhibitor, n (%)	14 (40.0)	31 (58.5)	0.139
Integrase Inhibitor, n (%)	7 (20.0)	9 (17.0)	0.939
$CD4^+$ T lymphocyte, cells/ml	731.17 ± 269.88	769.70 ± 375.62	0.695
$CD8^+$ T lymphocyte, cells/ml	930.43 ± 393.07	1038.65 ± 495.67	0.424
CD4/CD8 ratio	0.83 ± 0.29	0.77 ± 0.39	0.247
Cardiovascular risk factors			
Hypertension, n (%)	2 (5.6)	15 (27.3)	**0.020**
Type 2 diabetes mellitus, n (%)	3 (8.3)	4 (7.3)	1.000
Dyslipidemia, n (%)	2 (8.3)	7 (33.3)	0.086
Smoker, n (%)	14 (41.2)	25 (45.5)	0.861
Alcohol, n (%)	15 (42.9)	13 (25.5)	0.146
Cocaine drugs user, n (%)	6 (17.1)	4 (7.3)	0.268
Heroin drugs user, n (%)	1 (4.2)	6 (28.6)	0.066
Framingham risk score	2.63 ± 3.127	7.66 ± 5.84	**0.008**
Atherosclerosis assesment			
Carotid plaques, n (%)	0 (0.0)	10 (18.2)	**0.018**
cIMT left bulb carotid, mm	0.72 ± 0.13	1.09 ± 0.33	< **0.001**
cIMT left common carotid, mm	0.58 ± 0.10	0.77 ± 0.19	< **0.001**

Ab: antibodies; cIMT: carotid Intima Media Thickness; ART: Antiretroviral therapy; NNRTI: Non-nucleoside retroviral transcriptase inhibitor.

subclinical atherosclerosis compared with those patients with lower numbers (OR = 4, CI95% 1.19–13.3, p = 0.024). Consequently, 33% of patients with subclinical atherosclerosis showed numbers of $CD8^+CD28^-$ T cells higher than 59% compared with 13.9% of patients without atherosclerosis (p = 0.038). This cut-off had a sensitivity of 33.3% (CI95% 22.2–46.6), specificity of 86.1% (CI95% 71.3–93.9), positive predictive value of 78.3% (CI95% 58.1–90.3), and negative predictive value of 46.3% (CI95% 34.9–58.1) to detect subclinical atherosclerosis. Interestingly, 27.4% of patients with low Framingham risk score had elevated levels of $CD8^+CD28^-$ T cells compared to 26.7% of patients with elevated Framingham risk score.

Patients on abacavir (n = 18) compared with those without this treatment had higher absolute numbers of $CD8^+CD28^-$ T cells (402, 291–619 cell/μl vs. 304, 216–431 cell/μl, p = 0.05) and absolute numbers of $CD4^+CD28^-$ T cells (13.26, 3.37–24.16 cell/μl vs. 5.4, 3.11–11.7 cell/μl, p = 0.05). Patients on abacavir also had higher frequency of $HLA\text{-}DR^+$ NK cells (28.41%, 15–37.9% vs. 17.6%, 7.8–28.6%, p = 0.025), and higher absolute numbers of $CD8^+HLA\text{-}DR^+$ T cells (244.7, 113.1–292.3 cell/μl vs. 135.7, 80.0–232.0 cell/μl, p = 0.04).

Association of immunologic variables was analyzed in a multivariable analysis (adjusted for age, sex, HIV-viral load, Framingham risk score, CD4/CD8 ratio, percentage of $CD8^+HLA\text{-}DR^+$ T cells, and percentage of CD38 + CD19 B cells) to show that the percentage of $CD8^+HLA\text{-}DR^+$ T cells (OR = 1.044, CI95% 1.003–1.087, p = 0.036) and the ratio of CD4/CD8 T cells (OR = 0.19, CI95% 0.04–0.93, p = 0.041) were independently associated with the elevation of $CD8^+CD28^-$ T cells (Table 4). Significant direct correlation was found between the numbers of $CD8^+CD28^-$ T cells and the numbers of $CD8^+HLA\text{-}DR^+$ T cells (Pearson = 0.320, p = 0.02). Significant inverse correlation was found between the numbers of $CD8^+CD28^-$ T cells and the ratio of CD4/CD8 T cells (Pearson = −0.404; p < 0.001) and the numbers of $CD19^+CD38^+$ B cells (r = −0.246; p = 0.019), respectively (Fig. 2).

We analyzed the correlation between age and HIV infection duration and the percentage of $CD8^+CD28^-$ T cells (r = 0.117; p = 0.272 and r = −0.148; p = 0.183), $CD4^+CD28^-$ T cells (r = 0.062; p = 0.561 and r = −0.157; p = 0.157), $CD4^+CD38^+$ T cells (r = −0.18; p = 0.087 and r = −0.13; p = 0.24), $CD8^+CD38^+$ T cells (r = −0.191; p = 0.07 and r = 0.135; p = 0.221), $CD4^+HLA\text{-}DR^+$ T cells (r = 0.142; p = 0.179 and r = −0.1; p = 0.365), and $CD8^+HLA\text{-}DR^+$ T cells (r = 0.65; p = 0.543 and r = −0.097; p = 0.382) and no significant correlations were found.

4. Discussion

This cross-sectional study of long-term ART treated HIV-infected patients with less than 200 viral copies/ml revealed that T cells activation/senescence biomarkers correlated positively with the presence of subclinical atherosclerosis, independently of age, sex and classical cardiovascular risk factors. Importantly, these cheap and easy available immunological parameters might be useful as predictive biomarkers of cardiovascular risk in virally controlled HIV-infected patients.

Table 2
Immunologic characteristics distributed by subclinical atherosclerosis.

Parameters (mean ± sd)	No atherosclerosis (N = 36, 39.5%)	Subclinical atherosclerosis (N = 55, 60.4%)	p
General blood cell subsets			
Leukocytes (millions/ml)	6.80 ± 3.00	6.58 ± 1.94	0.679
Lymphocytes (%)	31.97 ± 8.91	29.46 ± 9.87	0.222
Monocytes (%)	6.96 ± 1.48	7.26 ± 1.93	0.423
Neutrophils (%)	57.27 ± 9.71	59.76 ± 10.37	0.254
Eosinophils (%)	2.46 ± 1.68	2.26 ± 1.11	0.488
General lymphocyte subsets			
$CD3^+$ T lymphocytes (%)	59.93 ± 14.94	62.92 ± 16.51	0.383
$CD4^+$ T lymphocytes (%)	25.72 ± 8.63	24.61 ± 8.28	0.541
$CD8^+$ T lymphocytes (%)	32.20 ± 9.48	37.01 ± 15.52	0.099
$CD4^+CD8^+$ T lymphocytes (%)	0.50 ± 0.42	0.81 ± 0.79	**0.037**
$CD3^-CD19^-CD16^+$ NK lymphocytes (%)	9.44 ± 5.83	10.10 ± 7.59	0.658
$CD19^+$ B lymphocytes (%)	10.17 ± 5.94	9.78 ± 5.00	0.739
Expression of activation markers in $CD4^+$ T lymphocytes			
$CD28^+$ (%)	89.55 ± 12.07	89.96 ± 8.62	0.850
$CD28^-$ (%)	10.44 ± 12.07	10.05 ± 8.62	0.859
$CD38^+$ (%)	50.67 ± 15.68	48.19 ± 11.70	0.389
HLA-DR+ (%)	13.23 ± 9.87	15.09 ± 9.68	0.376
$CD86^+$ (%)	3.87 ± 5.57	5.99 ± 7.53	0.153
CD28 (MFI)	2755 ± 1271	3253 ± 1437	0.096
Expression of activation markers in $CD8^+$ T lymphocytes			
$CD28^+$ (%)	58.29 ± 16.95	49.78 ± 16.17	**0.018**
$CD28^-$ (%)	41.70 ± 16.96	50.22 ± 16.15	**0.018**
$CD38^+$ (%)	38.18 ± 20.17	34.89 ± 16.78	0.402
HLA-DR+ (%)	21.20 ± 12.84	24.32 ± 12.90	0.261
$CD86^+$ (%)	3.74 ± 4.67	4.66 ± 4.54	0.356
CD28 (MFI)	1865 ± 789	2243 ± 917	**0.046**
Expression of activation markers in $CD4^+CD8^+$ T lymphocytes			
DR+ (MFI)	**2084.8 ± 2408.7**	**3152.4 ± 3696.5**	**0.296**
$CD38^+$ (MFI)	**947.9 ± 1051.9**	**829.9 ± 542.1**	**0.582**
$CD86^+$ (MFI)	**402 ± 791.14**	**481 ± 945.7**	**0.967**
$CD28^+$ (MFI)	**2153.07 ± 1303.1**	**2675.15 ± 1486.3**	**0.271**
Expression of activation markers in $CD3^-CD19^-CD16^+$ NK lymphocytes			
$CD8^+$ (%)	24.87 ± 14.71	32.30 ± 17.99	**0.042**
$CD38^+$ (%)	87.97 ± 9.13	87.03 ± 11.57	0.682
$CD86^+$ (%)	2.93 ± 3.40	5.27 ± 6.58	0.052
HLA-DR+ (%)	18.15 ± 13.14	23.36 ± 14.78	0.089
$CD38^+$ (MFI)	7639 ± 4988	7617 ± 4174	0.981
Expression of activation markers in $CD19^+$ B lymphocytes			
$CD38^+$ (%)	65.38 ± 27.47	42.67 ± 30.26	< **0.001**
$CD86^+$ (%)	9.38 ± 9.17	11.21 ± 12.97	0.466
HLA-DR+ (MFI)	15425 ± 7793	16681 ± 8213	0.470
Expression of activation markers in Monocytes			
CD38 (MFI)	5000 ± 1327	4891 ± 1130	0.675
CD86 (MFI)	1681 ± 1008	1570 ± 437	0.477
HLA-DR (MFI)	8655 ± 5071	9725 ± 5674	0.363

MFI: Mean Fluorescence Intensity.

In patients on ART, HIV infection not only mediates immune cell activation and endothelial dysfunction, but also activates an array of cellular pathways, such as inflammasome formation/caspase-1 activation, autophagy, oxidative stress, and endoplasmic reticulum stress (Kearns et al., 2017). Along with these mechanisms, ART therapy itself, HIV-associated comorbidities, such as dyslipidemia, drug abuse, opportunistic infections, and lifestyle, contribute to the development of atherosclerosis (Kearns et al., 2017). Besides, macrophage and T cell activation has been associated with the inflammatory process and plaque formation in HIV-associated atherosclerosis (Grome et al., 2017). Activated T cells are recruited along with macrophages into the endothelium where they produce proatherogenic mediators (Chow et al., 2016). Our results show that, even in long-term ART treated virally controlled patients, a differential activation and immune-modulation can be detected in patients developing subclinical atherosclerosis, which extend mainly to $CD8^+$ T cells, NK cells and B lymphocytes, but not to peripheral blood monocytes. In line with our results, it has been described that $CD8^+$ T-cell activation, but not monocyte activation, is associated with subclinical carotid artery disease in stable ART HIV-patients (Longenecker et al., 2013). Nonetheless, more recent data sustain that non-classical monocytes can predict progression of carotid artery bifurcation intima-media thickness, as well as coronary artery calcium progression in these patients (Chow et al., 2016; Zungsontiporn et al., 2016).

Chronic T-cell activation has been strongly correlated with atherosclerosis in several studies (Kearns et al., 2017; Kaplan et al., 2011; Grome et al., 2017). Even in the latent state with very low or undetectable viremia, viral regulatory proteins (Tat, Nef, and others) are produced in T cells and monocytes, which can alter their function (Kearns et al., 2017; Crowe et al., 2010). In line with our results, it has been shown that virally controlled HIV-infected patients and even HIV elite controllers without measurable viremia have abnormally high T-cell activation levels (Kaplan et al., 2011; Karim et al., 2014). Our results show higher expression of CD28-MFI on $CD8^+CD28^+$ T cells, higher frequency of double $CD4^+CD8^+$ T cells, but lower frequency of $CD38^+$ B lymphocytes in patients with subclinical atherosclerosis, which may be a manifestation of a more active anti-viral response mediated by $CD8^+CD28^+$ T cells (Blanco-García et al., 2011) and by the multifunctional $CD4^+CD8^+$ T cells (Frahm et al., 2012), as well as a possible B-cell dysfunction induced by the HIV-infection (Moir and Fauci, 2008, 2009), since CD38 receptors contributes to B-cell proliferation, rescue from apoptosis, and activation (Funaro et al., 1997).

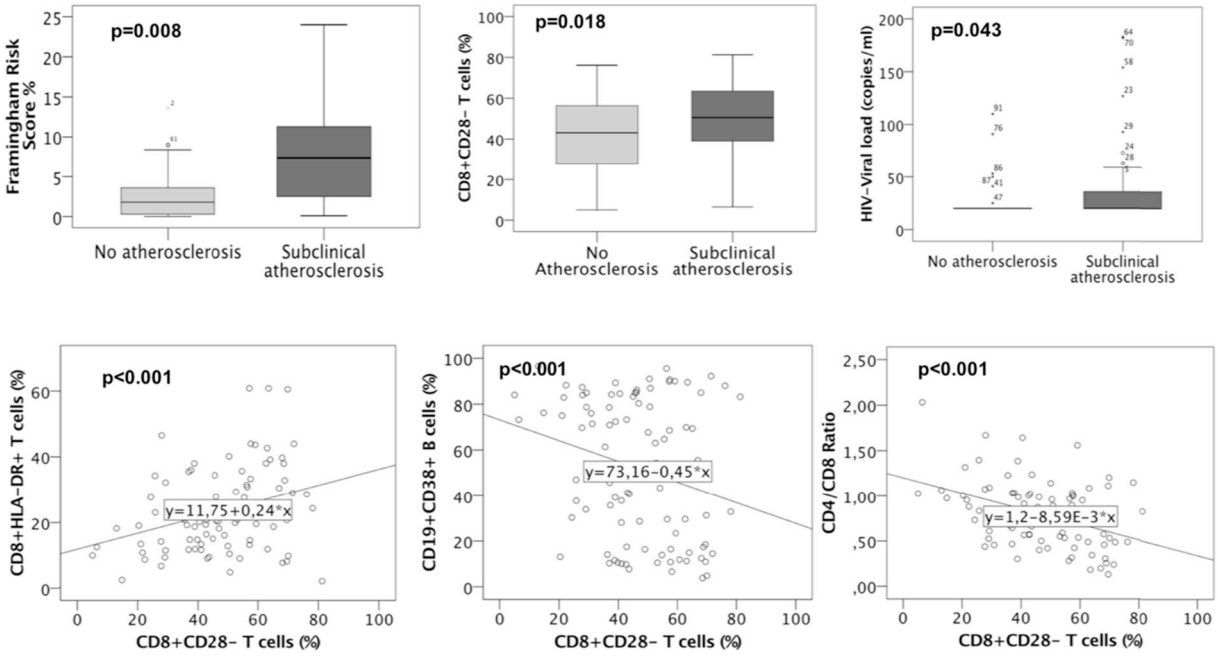

Fig. 2. Association of clinical and immunologic variables. Box plot of the percentage of $CD8^+CD28^-$ T cells, the Framingham risk score, and the HIV viral load according to presence of subclinical atherosclerosis (n = 55) vs. no atherosclerosis (n = 36). Correlation of the percentage of $CD8^+CD28^-$ T cells with the percentage of CD8 + HLA-DR + T cells, the ratio CD4:CD8 T cells, and the percentage of $CD19^+CD38^+$ B cells (%).

Table 3
Multivariable analysis of variables associated with subclinical atherosclerosis.

	Multivariable analysis		
	OR	95%CI	p
Intravenous drugs use	0.263	0.077–0.893	0.032
PI regimen	0.482	0.166–1.4	0.181
HIV viral load	2.396	0.575–9.98	0.23
Framingham risk score > 10%	**14.84**	**1.63–125**	**0.016**
$CD28^-CD8^+$ T cells (%)	**1.032**	**1–1.065**	**0.045**
CD4/CD8 ratio	1.093	0.216–5.43	0.914

PI: protease inhibitor.

Table 4
Multivariable analysis of variables associated with the elevation of $CD8^+CD28^-$ T cells.

	Multivariable analysis		
	OR	95%CI	p
Age	1.038	0.973–1.1	0.259
Sex	0.466	0.102–2.12	0.324
HIV viral load	1.004	0.989–1.019	0.573
Framingham risk score > 10%	0.169	0.025–1.16	0.072
$CD8^+$HLA-DR^+T cells	**1.044**	**1.01–1.087**	**0.036**
CD4/CD8 Ratio	**0.196**	**0.041–0.937**	**0.041**
$CD19^+CD38^+$B cells	0.99	0.9711.01	0.335

Supporting these results, and although all patients in our series showed HIV counts below 200 copies/ml, patients with subclinical atherosclerosis showed slightly but significantly higher HIV copies/ml. Nonetheless, it is not possible to rule out that, behind this differential T cell activation, other factors such as concomitant viral infections or gastrointestinal bacterial translocation could be involved (Slim and Saling, 2016).

Chronic immune activation also leads to gradual accumulation of highly-differentiated, antigen-specific, oligoclonal T cells, particularly within the $CD8^+$ T-cell compartment (Strioga et al., 2011). These cells are characterized by critically shortened telomeres, loss of CD28 and/or gain of CD57 expression. These $CD8^+CD28^-$ (and/or $CD8^+CD57^+$) T cells, which comprise functionally competing cytotoxic, pro-inflammatory, suppressive/regulatory and senescent subsets (Strioga et al., 2011), appear to play a significant role in various diseases associated with chronic immune activation, including elevated risk of atherosclerotic vascular disease in ART treated HIV patients (Longenecker et al., 2013; Strioga et al., 2011). Along with $CD8^+CD28^-$ T cells, HIV chronic immune stimulation leads to $CD4^+CD28^-$ T cell accumulation. $CD4^+CD28^-$ T cell counts has prognostic significance for myocardial infarction or death in non-HIV subjects with recurrent ischaemic coronary episodes (Liuzzo et al., 2007), and can be isolated from ruptured atherosclerotic plaques. This finding has been interpreted as evidence that $CD4^+CD28^-$ T cells are involved in the development of unstable plaques (Zal et al., 2004). In our study, no differences between patients with or without subclinical atherosclerosis were found regarding activated ($CD38^+$ and/or DR^+) or senescent ($CD28^-$) $CD4^+$ T cells, possibly because treatments in our patients extended for more than 9 years (25% of them for more than 20 years) and all patients had very low HIV copies. In contrast, higher $CD8^+CD28^-$ T cell values were detected in patients with subclinical atherosclerosis, a T cell subset that appears to be most directly associated to atherosclerosis in long-term treated HIV patients (Longenecker et al., 2013). Interestingly, neither HIV infection duration nor patient's age in our series were related to a higher degree of immune activation or immune senescence. However, the direct correlation between the frequency of $CD8^+CD28^-$ and $CD8^+$HLA-DR^+ T lymphocytes and the inverse correlation with the ratio of CD4/CD8 T lymphocyte suggest that a link between immune senescence and immune activation could exist, and reinforces the hypothesis that these biomarkers could be related to atherosclerosis in long-term ART treated HIV patients (Serrano-Villar et al., 2014a).

In line with previous reports (Bernal et al., 2011; Serrano-Villar et al., 2014b), our results also show that long-term ART-treated stable HIV patients have an increased risk of subclinical atherosclerosis, even having low cardiovascular risk. In our series, 60% of patients had subclinical atherosclerosis despite the fact that only 18% of them had high cardiovascular risk according to Framingham risk score. Although

many factors are involved in the pathogenesis of atherosclerosis, most of them related to the classic cardiovascular risk factors represented on the Framingham scale, new underlying mechanisms that could explain its increased prevalence in HIV patients need to be explored. Our results, along with those described by Longenecker et cols. (Longenecker et al., 2013), shed light by pointing to the chronic HIV induced immune activation and immune senescence, with $CD8^+CD28^-$ T cells playing a potential role. On the other hand, it is interesting to note that approximately 30% of patients with low cardiovascular risk according to Framingham risk score had high levels of $CD8^+CD28^-$ T cells, so they would be erroneously classified as low risk when they are high risk patients. This result is in favor of the use of other prognostic scales of cardiovascular risk such as D:A:D adapted for HIV infected patients who have shown a better predictive capacity in these patients (Markowicz et al., 2014).

Besides, an interesting finding of our study was that patients on abacavir treatment had higher immunosenescence (increased figures of $CD8^+CD28^-$ and $CD4^+CD28^-$) compared to those who took another regimen. In addition, they presented higher immunoactivation markers such as HLA-DR on NK cells and $CD8^+$ T cells. Although these findings were blurred in the multivariate analysis, they could serve as a basis for future research and help to clarify the increased cardiovascular risk presented in these patients (Dorjee et al., 2018).

In conclusion, and despite the limitations of our study, mainly concerning the number of patients that hinder the significance of our results or its cross-sectional nature that difficult causality assignment of observed associations, our results show that immune activation, and especially immune senescence, represented by the accumulation of $CD8^+CD28^-$ T cells, could constitute important mechanisms involved in the higher prevalence and progression of subclinical atherosclerosis in stable virally controlled HIV-infected patients. Although further studies with larger series are needed to verify these results, this easy available immunological parameter might be useful as predictive biomarker of cardiovascular risk especially in patients with low Framingham risk score and high number of $CD8^+CD28^-$ T cells who could benefit from early personalized attention and the administration of a more intensive treatment.

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